

Tip60 Is Required for DNA Interstrand Cross-link Repair in the Fanconi Anemia Pathway*

Received for publication, November 6, 2007, and in revised form, February 5, 2008 Published, JBC Papers in Press, February 8, 2008, DOI 10.1074/jbc.M709076200

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The disease Fanconi anemia is a genome instability syndrome characterized by cellular sensitivity to DNA interstrand cross-linking agents, manifest by decreased cellular survival and chromosomal aberrations after such treatment. There are at least 13 proteins acting in the pathway, with the FANCD2 protein apparently functioning as a late term effector in the maintenance of genome stability. We find that the chromatin remodeling protein, Tip60, interacts directly with the FANCD2 protein in a yeast two-hybrid system. This interaction has been confirmed by co-immunoprecipitation and co-localization using both endogenous and epitope-tagged FANCD2 and Tip60 from human cells. The observation of decreased cellular survival after exposure to mitomycin C in normal fibroblasts depleted for Tip60 indicates a direct function in interstrand cross-link repair. The coincident function of Tip60 and FANCD2 in one pathway is supported by the finding that depletion of Tip60 in Fanconi anemia cells does not increase sensitivity to DNA cross-links. However, depletion of Tip60 did not reduce monoubiquitination of FANCD2 or its localization to nuclear foci following DNA damage. The observations indicate that Fanconi anemia proteins act in concert with chromatin remodeling functions to maintain genome stability after DNA cross-link damage.

Fanconi anemia (FA)² is a rare disease arising from a defect in any of at least 13 proteins. The disease is characterized by malformations, pancytopenia of bone marrow, and an increased risk of leukemias and solid tumors (1). The hallmark of Fanconi anemia at the cellular level is a pronounced hypersensitivity to DNA interstrand cross-linking (ICL) agents; this hypersensitivity is manifested as an elevated number of chromosomal breaks and radial formations as well as decreased cell survival. A core complex of FANCA proteins that includes FANCA, FANCB, FANCC, FANCE,

FANCE, FANCG, and FANCL (1–4) is required for the monoubiquitination of FANCD2 (FANCD2-Ub) after exposure of cells to ICL agents, ionizing radiation, UV irradiation, or replication fork stalling. The post-translational modification of FANCD2 is required for localization of FANCD2 to damage-induced foci in the nucleus and for FA pathway function maintaining normal genome stability after ICL formation (5). It appears that FANCD2-Ub localizes to chromatin in the nuclear foci (6, 7), and it may be that the localization to chromatin is essential for function of the FA pathway. The recently identified FANCI protein undergoes a similar monoubiquitination, and it appears FANCD2-Ub and FANCI-Ub act cooperatively as a dimer (8).

Remodeling of chromatin structure surrounding DNA damage appears to be required for optimal DNA repair and may be needed for efficient loading of proteins involved in DNA repair after several types of DNA damage, including double strand breaks (DSB) (7, 9, 10). For example, histone H2AX is modified by phosphorylation (γ H2AX) by DNA-PK, ATR, or ATM kinases, following DNA damage or strand breaks (11, 12). The γ H2AX accumulates around strand breaks in megabase regions (13) and may help recruit other repair factors (14). γ H2AX also localizes to the nuclear foci induced by DNA damage (12). Moreover, mice lacking γ H2AX are hypersensitive to ionizing radiation (15). Thus there is evidence that chromatin modifications are directly involved in DNA repair and genome stability.

Tip60, a histone acetyltransferase (16) that was first identified as a human immunodeficiency virus Tat-interacting protein (17), has been implicated in DSB repair (18) and as a co-regulator of transcription for a number of proteins, including p53, c-Myc, and others (reviewed in Refs. 19 and 20). The acetyltransferase site resides in a conserved motif in the C-terminal region of the protein. Tip60 is a component of a conserved chromatin remodeling complex (21, 22), which is homologous to the *Saccharomyces cerevisiae* NuA4 complex. The yeast homolog of Tip60, Esa1, in the NuA4 complex, is essential for viability. The *Drosophila* homolog of Tip60 is involved in acetylation and exchange of histones surrounding DSBs (23). In addition to histones, Tip60 acetylates several target proteins that are involved in DNA repair or checkpoint responses to DNA damage, including p53 (23) and ATM (24). Tip60 also co-localizes with γ H2AX in DNA damage-induced foci (24). Recently Tip60 has been demonstrated to acetylate H2AX and thereby regulate its ubiquitination by UBC13 during DSB repair (25). Thus Tip60 is a chromatin remodeling protein for which there is evidence of function in genome stability following DNA damage.

* This work was supported by NHLBI, National Institutes of Health Program Project Grant 1P01HL48546 and Northwest Health Foundation Grant 2001-218. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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² The abbreviations used are: FA, Fanconi anemia; ICL, interstrand cross-linking; DSB, double strand break(s); MMC, mitomycin C; GST, glutathione S-transferase; PBS, phosphate-buffered saline; siRNA, small interfering RNA; X- α -Gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside.

As a result of a yeast two-hybrid screen for proteins that interact with FANCD2, we identified Tip60 as a FANCD2-interacting protein. The interaction was confirmed by co-immunoprecipitation and co-localization of Tip60 and FANCD2 in damage-induced foci. Mutagenesis of the acetyl-CoA-binding site of Tip60 abrogates the interaction with FANCD2, but FANCD2 does not require monoubiquitination to interact. To facilitate monitoring the interaction, epitope-tagged FANCD2 constructs were used in some instances, after demonstrating the functionality of the constructs by complementation of *Fancd2* cells for genome stability following mitomycin C (MMC) treatment. Depletion of Tip60 sensitizes cells to the DNA cross-linking agent MMC, but depletion of Tip60 in FA cells does not further sensitize them to ICL agents. Depletion of Tip60 did not reduce formation of FANCD2-Ub or localization of FANCD2 to nuclear foci following ICL formation. Therefore the action of Tip60 must not be in the function of the FA core required for FANCD2-Ub formation. We conclude that Tip60 functions in DNA interstrand cross-link repair, interacting with FANCD2, and that the action of Tip60 is epistatic to the FA pathway.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—A 2751-bp EcoRV-BglII fragment covering amino acid 55–971 of FANCD2 cloned into the SmaI and BamHI sites of pGBKT7 (Clontech), respectively, was transformed into yeast strain AH109 (Clontech), which was mated with yeast strain Y187 (Clontech) pretransformed with a human fetal brain cDNA library. Approximately 2×10^6 diploid colonies growing on complete medium were plated onto synthetic dropout plates lacking tryptophan, leucine, and histidine. Approximately 300 colonies that arose under this triple selection were then streaked onto synthetic dropout plates lacking tryptophan, leucine, histidine, and adenine supplemented with 1 mM 3-amino-1,2,4-triazole. Approximately 50 colonies survived this quadruple selection, and plasmids were recovered and sequenced. After rejection of genomic clones and out-of-frame sequences, candidate plasmids were retransformed into Y187 and mated with AH109 carrying pGBKT7-FANCD2(55–971) to confirm the interactions by plating on synthetic dropout plates lacking tryptophan, leucine, and histidine, with X- α -Gal (Clontech) as a reporter of β -galactosidase induction. The cell extracts were also assayed for β -galactosidase activity using O-nitrophenyl- β -D-galactopyranoside (Sigma), following the yeast two-hybrid Matchmaker protocol (Clontech).

Plasmids and Constructs—Full-length Tip60 cDNA was amplified from a human lymphocyte cDNA library (a gift from Manual Buchwald) with primers Tip60-1 (5'-CACAGAATTC-GGGAAGATGGCGGAGGTGG) and Tip60-2 (5'-CACACTCGAGTCACCACTTCCCCCTCTTGC) and cloned into pCMVTag-2B (Stratagene) to generate a FLAG-tagged construct. Tip60 was subcloned into pGex6P-1 (GE Healthcare), pGADT7, and pGBKT7 (Clontech). All three known splice variants of Tip60 were obtained as individual clones, verified by sequencing. Full-length FANCD2 cDNA was subcloned into pAc5.1 (Invitrogen), and the C-terminal V5 and His₆ epitope tags, along with the FANCD2 coding sequence, were subcloned

into pMMPpuro as pMMPpuro-FANCD2-V5His. For N-terminal epitope-tagged FANCD2, pMMPpuro was modified by adding a synthetic multicloning site that included restriction sites for Eco47III and XhoI. The His₆ and V5 cassette was then subcloned into the modified pMMPpuro vector, via NcoI and Eco47III, to yield pMMPpuro-HV. The FANCD2 coding sequence was then amplified, using primers FAD-47III (5'-CACAAGCGCTATGGTTTCCAAAAGAAGACTGTC) and FAD-17 (5'-CACACTCGAGCTAATCAGAGTCATAACTCTC) and cloned into the Eco47III and XhoI sites of pMMPpuro-HV. Pirh2 coding sequence was amplified from a human fetal limb cDNA library using primers pirh2-F (5'-CACAGAATTCATGGCGGCGACGGCCCGG) and pirh2-R (5'-CACAGGATCCTTGCTGATCCAGTGAAT), cloned into pGADT7 (Clontech). Control pGADT7-FANCA was described previously (26). Control FANCG coding sequence was amplified with primers MEM-13 (5'-CACAGAGGCCAGTGAATTCATGTC) and MEM-12 (5'-CACAGTCGACCAGGTCACAAGACTTTGGCAGAG, digested with EcoRI and SalI, and cloned into pBridge (Clontech). Routine cloning was with *Escherichia coli* XL1-Blue MRF' cells (Stratagene).

Immortalized Human Fibroblast Cells—Immortalized GM639, HEK293, GM6914 (FA-A) (NIGMS, National Institutes of Health Human Genetic Cell Repository), PD20 (FA-D2), and PD331 (FA-C) (Oregon Health & Science University Fanconi Anemia Cell Repository) cells were cultured in α -minimum Eagle's medium (Mediatech) supplemented with 5% fetal bovine serum (Hyclone), 5% calf serum (Hyclone), and 0.1% gentamicin (Invitrogen) in a humidified incubator with 5% CO₂ at 37 °C. To generate cell lines stably expressing epitope-tagged FANCD2, the pMMPpuro-FANCD2-V5His construct was packaged in AM12 cells (ATCC), and the viral supernatant was used to transfect PD20 cells. Puromycin-resistant clones were evaluated for functional complementation as defined by normal levels of radial formations after treatment with MMC.

Antibodies, Immunoblotting, and Immunoprecipitations—Anti-Tip60 (N-17) (Santa Cruz Biotechnologies), anti-V5 (Invitrogen), monoclonal anti-FANCD2 (Santa Cruz), anti-Tip49 (Abcam), anti-c-Myc (Santa Cruz), anti-FLAG (Sigma), and anti-hemagglutinin (Santa Cruz), were used according to the vendors' recommendations. The blots were visualized by Western Lightning ECL reagent or Millipore ECL reagent and exposure on x-ray film. The immunoblots were performed as described (30). Protein concentrations were determined by dye binding (Bio-Rad). For immunoprecipitations, ~1 mg of extract protein was precleared by adding 6.5 μ g of normal mouse serum and 50 μ l of protein A/G Plus agarose beads (Santa Cruz Biotechnology). After incubation for 1 h at 4 °C, the beads were removed by centrifugation. Immunoprecipitations with α -V5 used ~1 mg of precleared extract and 2.5 μ g of α -V5 antibody. After binding the α -V5 antibody overnight at 4 °C, 30 μ l of protein A/G Plus agarose beads were added, incubated 1 h at 4 °C, and then centrifuged 15 min at 3000 rpm at 4 °C. The beads were then washed three times with 500 μ l of lysis buffer, mixed gently for 5 min, and then centrifuged as before. The final bead pellet was resuspended in 80 μ l of loading dye consisting of 60 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.1 M dithiothreitol, and 0.001% bromophenol blue and then heated 3 min

Direct Interaction between FANCD2 and Tip60

at 85 °C, followed by centrifugation for 5 min at 3000 rpm to remove beads. The proteins were resolved on 7.5% denaturing acrylamide gels and transferred to polyvinylidene difluoride membranes for immunoblotting.

Immunoprecipitations of transiently expressed FLAG-tagged Tip60 from HEK293 cells with anti-FLAG antibody were carried out as for the anti-V5 antibody, and control immunoprecipitations were performed with extracts from nontransfected cells. Immunoprecipitation of endogenous FANCD2 from GM639 extracts was carried out as above, but monoclonal anti-FANCD2 antibody was used in place of anti-V5.

GST Fusion Pulldown—The GST fusion pulldown assay was adapted (27), as was the interaction assay (28). GST-Tip60 β and GST were grown in BL21 Codon Plus RIL cells (Stratagene) in 10 ml of LB and induced for 4 h with 2 mM isopropyl β -D-thiogalactopyranoside. The cells were harvested, washed in PBSE (PBS plus 1 mM EDTA), and lysed in 0.6 ml of PBSE supplemented with 100 μ g/ml lysozyme, chilled on ice 15 min, at which time dithiothreitol was added to 5 mM; phenylmethylsulfonyl fluoride was added to 1 mM with lysis by freeze-thawing, 0.6 ml of a 50% slurry of DE52 cellulose (Whatman) equilibrated in 0.3 M KPO₄, pH 7.0, was added, and the lysate was cleared by centrifugation. An additional 100 μ l of the DE52 slurry was added, mixed, and centrifuged as before. Triton X-100 in PBSE was added to a final concentration of 1.5% to the lysate. 130 μ l of a 50% slurry of GSH-Sepharose beads (GE Healthcare) equilibrated in PBSE were added and rotated 3 h at 4 °C. The beads were then washed three times with 0.5 ml of PBSE, pelleted by centrifugation at 2000 rpm, 5 min, 4 °C, and then resuspended in 65 μ l of PBSE. 10 μ l of the slurry were removed and analyzed by SDS-PAGE followed by Coomassie staining. Bound GST fusion protein concentrations were estimated from the Coomassie-stained gel, using a serial dilution of bovine serum albumin for a standard curve. Approximately 2 μ g of GST-Tip60 β and GST bound to GSH-Sepharose beads, respectively, were then transferred to clean microcentrifuge tubes and equilibrated in Buffer A (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Tween 20, 2 mM MgCl₂, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 \times Complete protease inhibitor mixture without EDTA (Roche Applied Science)) by centrifugation and resuspension in 100 μ l of Buffer A. Approximately 250 mg of GM639 cleared whole cell extract in Buffer A was added to each sample and rotated 2 h at 4 °C. The beads were then washed three times with 0.5 ml of Buffer A by centrifugation and removal of the supernatant. The final bead pellet was resuspended in 50 μ l of SDS-PAGE sample loading dye, and 20 μ l was analyzed by immunoblot.

Immunofluorescence—The cells were seeded onto glass coverslips. The following day, the cells were transfected with Tip60 siRNA. On day 2, the cells were treated with 80 ng/ml MMC (Sigma). After another 16 h, the cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The coverslips were then washed once with PBS and permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 15 min at room temperature. The coverslips were again washed with PBS and then blocked with 15% fetal bovine serum in PBS for 1 h at 37 °C. Blocking buffer was then replaced with anti-V5 and anti-Tip60 antibodies, diluted 1:500 and 1:100, respectively, in

blocking buffer, 1 h at 37 °C. The coverslips were then washed three times with PBS supplemented with 0.1% Tween 20 (Sigma), for 5 min each wash. Secondary antibodies (Texas Red donkey anti-mouse IgG (Abcam) and AlexaFluor 488 donkey anti-goat IgG (Invitrogen)), diluted 1:1000 in blocking buffer were then added and incubated 1 h at 37 °C. The coverslips were washed three times with PBS supplemented with 0.1% Tween 20 as before, followed by mounting of coverslips on slides with 30 μ l of Prolong Gold antifade reagent with 4',6'-diamino-2-phenylindole (Invitrogen) and curing 24 h in the dark. The slides were examined with a Nikon Eclipse 80i fluorescent microscope, and the images were captured with Elements Basic Research and Image-J (National Institutes of Health) software.

siRNA Transfection, DNA Damage, Cell Survival, and Cytogenetics—Transfections with plasmid DNA used Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Transfections with siRNA were performed as previously described (29). Controls were transfected with either a nonfunctional siRNA duplex or mock transfected. The transfection efficiency was monitored with transfections of labeled siRNAs (FAM *Silencer* siRNA labeling kit (Ambion)). For controls, a nonfunctioning duplex with the following sequence was used: AAC UUU UGC AAA GCG GAG CCA UU. Tip60 and Tip49 Smartpool siRNA and the nonfunctioning siRNA were from Dharmacon. Cell survival analysis was by colony survival, as described (29). Chromosome breakage analysis was performed by the Cytogenetics Core Lab at Oregon Health & Science University as previously described (29).

RESULTS

Yeast Two-hybrid Screen Identifies Tip60 as a FANCD2-interacting Protein—The yeast two-hybrid system allows precise definition of protein-protein interaction by prototrophic growth or reporter gene expression (β -galactosidase) because of translation of cDNA sequences fused to a binding domain sequence or transcriptional activation domain sequence when both domains are occupied (30). A yeast two-hybrid screen for FANCD2-interacting proteins was done using a FANCD2 N-terminal construct comprising amino acid 55–971 as bait, fused to the binding domain (Fig. 1A) and a commercially available human fetal brain cDNA library fused to the activation domain. Screening was done by mating cells carrying the FANCD2 bait with cells carrying the library and selecting for growth of the diploids on synthetic drop-out medium. Fifty ADE⁺ HIS⁺ clones obtained from screening two million clones were sequenced. After rejecting false positives, six clones, representing four candidate genes, remained. Tip60 was represented by two independent clones from different plates. The C-terminal 228 amino acids present in these clones included the catalytic acetyl-CoA-binding site of the acetyltransferase (Fig. 2B).

Confirmation of the FANCD2-Tip60 interaction was done by transformation of yeast with the rescued Tip60 clones and mating with yeast carrying the original FANCD2 (amino acids 55–971) bait. In addition, full-length Tip60 cDNAs (both Tip60 α and Tip60 β PLIP variants were used interchangeably, with the same results) were cloned into the activation domain

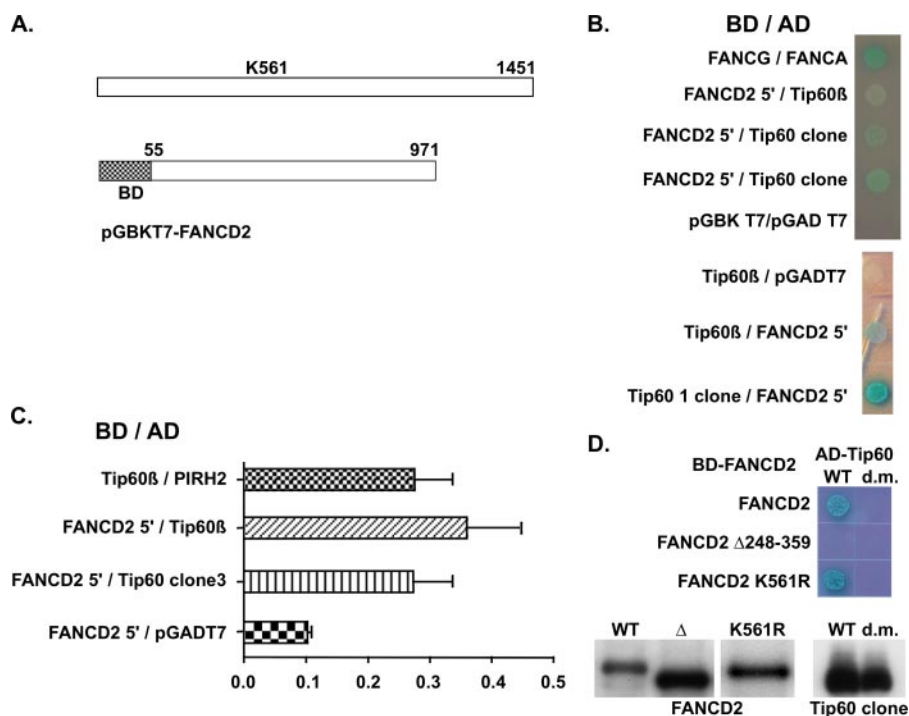


FIGURE 1. The FANCD2-Tip60 interaction is independent of FANCD2 Lys⁵⁶¹ but requires the Tip60 acetyl-CoA-binding site. *A*, subcloning of FANCD2 amino acid 55–971 into pGBKT7. *BD* is the binding domain. *B*, diploid yeast plated on –Leu–Trp–His–Ade with X- α -Gal. Library clones, as well as full-length Tip60 β , interact with FANCD2 in either binding or activation domain constructs (FANCD2–5' denotes the subclone encoding amino acid 55–971) as indicated by blue. The interaction of FANCG and FANCA is shown for comparison. *C*, liquid β -galactosidase assay of diploid yeast with indicated constructs. *D*, mutant proteins were expressed in diploid yeast, as shown by immunoblotting. FANCD2 and Tip60 fusions were probed with α -Myc and α -hemagglutinin, respectively, for the epitope tag fusion from the yeast vector. *WT*, wild type; *d.m.*, double mutant.

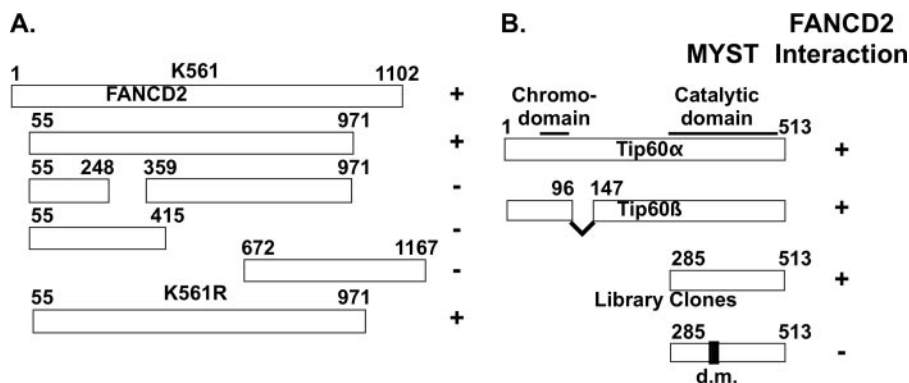


FIGURE 2. Interacting subclones. *A*, subclones of FANCD2, with interaction with Tip60 indicated as +. The K561R mutation prevents monoubiquitination of FANCD2. *B*, subclones of Tip60, with interaction with FANCD2 indicated as +. *d.m.*, double mutant.

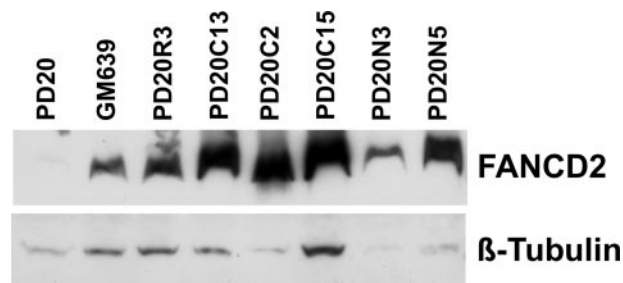


FIGURE 3. Immunoblot blot of PD20 clones transfected with FANCD2 expression constructs. In clone name, C indicates C terminus tag; N indicates N terminus tag. PD20R3 was transfected with a pIRESneo-FANCD2 nontagged construct. GM639 and PD20 whole cell extracts are shown for comparison.

of vector pGADT7 and tested with FANCD2 as bait. The rescued FANCD2-interacting clones, as well as the full-length Tip60 β constructs, interacted with FANCD2 as demonstrated by growth in the absence of adenine and histidine (Fig. 1*B*).

The FANCD2-Tip60 interaction was verified by switching the bait and prey, *i.e.* subcloning the FANCD2 insert into the activation domain and the Tip60 clones into the binding domain (Fig. 1*B*), again producing prototrophic growth. The reporter gene β -galactosidase was also induced on X- α -Gal plates (Fig. 1*B*). Interaction of FANCA and FANCG was done for comparison, because this interaction is well established (26, 31). The interaction was also indexed by liquid β -galactosidase assays (Fig. 1*C*) and showed a level of induction comparable with Pirh2-Tip60, a known Tip60 interaction (32). Because the original interacting clones were partial cDNA sequences carrying the C-terminal half of Tip60, the results demonstrate that the FANCD2-interacting domain resides in the C-terminal half of Tip60.

There are no FA homologs in yeast, and we have not observed evidence of FANCD2-Ub, with or without DNA damage, in yeast extracts. Immunoblotting of FANCD2 constructs from yeast cells showed a single band (Fig. 1*D*). Therefore, as expected, site-directed mutagenesis of FANCD2 lysine 561 (the site of FANCD2 monoubiquitination) to arginine did not alter the interaction with Tip60 (Fig. 1*D*). We also tested whether the acetyl-CoA-binding

site of Tip60 was essential for the interaction with FANCD2. Two critical amino acids, asparagine 377 and glycine 380, were mutated to glutamate. These alterations abrogated the FANCD2-Tip60 interaction (Figs. 1*D* and 2*B*). The same mutations have been shown to impede DSB repair and apoptosis in human cells (18).

Additional subclones of FANCD2 were tested for the ability to interact with Tip60 (Fig. 2). Deletion of exons 10–13 of FANCD2 (amino acid 248–359) eliminated the interaction (Fig. 1*D*).

FANCD2 subclones spanning amino acid 55–415 and 672–1167 also failed to interact with Tip60, thus localizing the Tip60-interacting region of FANCD2 to amino acid 248–672.

Direct Interaction between FANCD2 and Tip60

Thus the FANCD2-Tip60 interaction depends on an intact acetyl-CoA-binding site, part of the acetyltransferase catalytic site, but does not require FANCD2-Ub.

Epitope-tagged FANCD2 Protein Is Functional—As a useful aid for immunoprecipitation studies, we made epitope-tagged FANCD2 constructs. Retroviral expression constructs incorporating the V5 and His₆ epitope tags at either the N or C terminus of FANCD2 were packaged, and the viral supernatants were used to transfect PD20 (*fancd2*) cells. After stable transfectants

TABLE 1

Cytogenetic analysis of stably transfected PD20 clones

The cells were treated with 40 ng/ml MMC. PD20-3-15 is a clone that was complemented by microcell-mediated chromosome transfer of chromosome 3. The radials columns shows the percentage of cells having at least one radial.

PD20 Clone	Radials
	%
PD20-3-15 (microcell fusion)	12
PD20R3 (no tag)	3
PD20C13 (C-terminal tag)	10
PD20N3 (N-terminal tag)	14
PD20C15 (C-terminal tag)	56
PD20N5 (N-terminal tag)	67

were selected, individual clones showed varying degrees of FANCD2 expression (Fig. 3). This was easily monitored because PD20 does not manifest significant FANCD2 by immunoblot (Fig. 3). The clones were screened for FANCD2 function by scoring chromosomal aberrations after treatment with MMC. A number of independent, functionally complemented clones with C-terminal or N-terminal tagged FANCD2 protein were obtained. The genome stability of the clones after MMC treatment was comparable with normal cells or a PD20 derivative that had been complemented by microcell-mediated chromosome transfer (33) (Table 1). The overall efficiency of stable puromycin clone derivation was very low compared with other transfections based on the viral vector, approximately a thousand-fold lower. This observation is compatible with exogenous expression of FANCD2 being toxic to the cell. In an alternate approach to obtaining a complemented PD20 line, using a pIRES-FANCD2 plasmid, one stable expressing, corrected cell clone was obtained. Although without epitope, the level of expression was comparable with or greater than the viral transfectants (Fig. 3).

Several clones were selected, subcloned, and remained com-

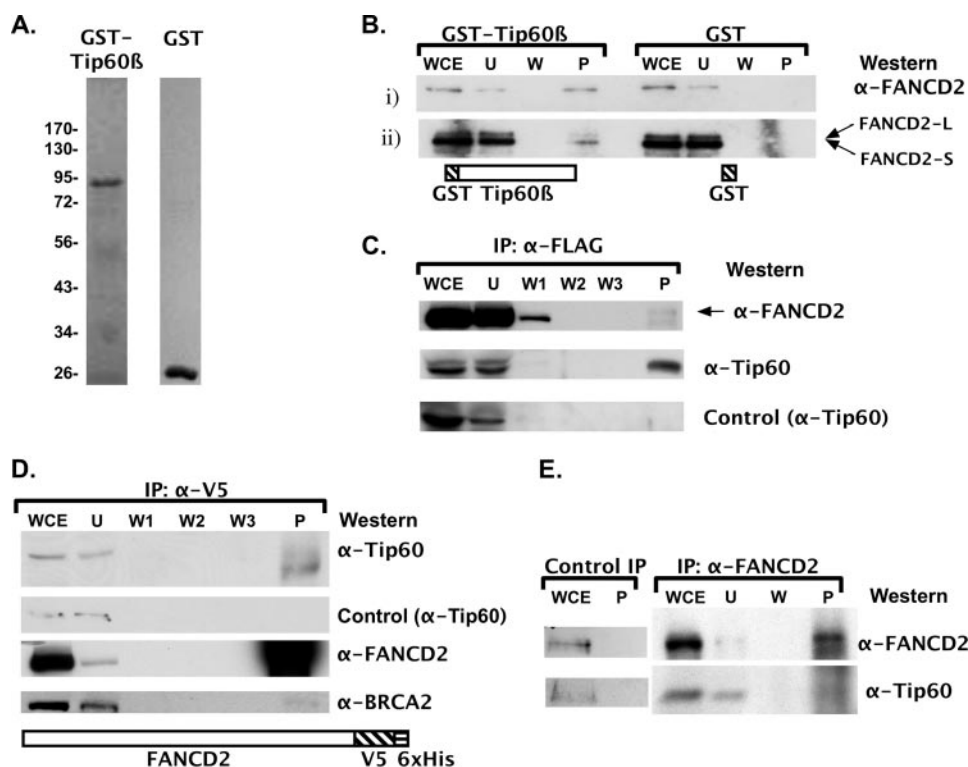


FIGURE 4. FANCD2 and Tip60 co-immunoprecipitate. A, GST-Tip60β and GST alone bound to glutathione-agarose beads, Coomassie-stained. B, GST-Tip60β pulldown of FANCD2. Lane WCE, GM639 cleared whole cell lysate; lane U, unbound fraction; lane W, wash 3; lane P, fraction bound to beads. Electrophoresis on 8% SDS-PAGE (panel i) demonstrated pulldown of FANCD2, but did not separate FANCD2-Ub (FANCD2-L) from unmodified FANCD2 (FANCD2-S). Electrophoresis on a 3–8% denaturing gradient gel (panel ii) resolved the two forms. C, co-immunoprecipitation (IP) of FANCD2 with FLAG-tagged Tip60. HEK293 cells were transiently transfected with FLAG-Tip60, harvested, and immunoprecipitated with anti-FLAG antibody and protein A/G-agarose beads. Lane WCE, input HEK293 whole cell cleared lysate; lane U, unbound fraction; lanes W1, W2, and W3, successive washes; lane P, immunoprecipitate. The control was an anti-FLAG immunoprecipitation with non-transfected cell extract. D, co-immunoprecipitation of BRCA2 and Tip60 with FANCD2-V5His. PD20 cells functionally complemented with FANCD2-V5His, were lysed and immunoprecipitated with α-V5 antibody and protein A/G-Sepharose beads. Lane WCE, cleared lysate; lane U, unbound; lanes W1, W2, and W3, washes 1, 2, and 3, respectively; lane P, final pellet. Control was the same anti-V5 immunoprecipitation with PD20 (FA-D2) nontransfected whole cell extracts, probed with anti-Tip60 antibody. E, co-immunoprecipitation of Tip60 with anti-FANCD2 antibody. Immunoprecipitations from whole cell extracts used normal mouse serum (control) or anti-FANCD2 antibody, and the blots were probed with anti-FANCD2 and anti-Tip60 antibody.

plemented after serial passage. Complementation did not correlate with apparent FANCD2 expression levels, as noted by immunoblot (Fig. 3). Two of the selected complemented clones and one uncomplemented clone were analyzed by DNA sequence analysis and found to be normal for rescued sequence and showed FANCD2 protein by immunoblot. Identification of active FANCD2, defined by cellular complementation, indicates that FANCD2 can tolerate the addition of a short peptide to either terminus without impairing function.

FANCD2 and Tip60 Co-immunoprecipitate—As a second approach to confirm the FANCD2-Tip60 interaction, pulldown strategies were employed. Full-length Tip60β was subcloned into a GST fusion vector (Fig. 4A). The fusion protein bound to GSH beads was then mixed with whole cell extracts from human fibroblasts. After extensive washing, the beads were resuspended and separated by SDS-PAGE. Immunoblots showed endogenous FANCD2 protein bound to the GST-Tip60β beads but not to control GST beads (Fig. 4B, panel i). Because the Tip60-FANCD2 interaction in the yeast two-hybrid system was independent of the ubiquitination status of FANCD2, we asked whether the interaction in

human cells might favor the nonubiquitinated form of FANCD2 over the monoubiquitinated form. As shown (Fig. 4B, *panel ii*), the GST-Tip60 β pulled down both forms of FANCD2, in close approximation to the ratio in the whole cell extract (FANCD2-L/FANCD2-S = 0.4 in the whole cells extract, 0.3 in the pellet fraction), indicating no preference for either form.

Epitope tags were used in co-immunoprecipitation experiments. FLAG-tagged Tip60 immunoprecipitated from whole cell extracts of transiently transfected HEK293 cells was performed, as was the reciprocal co-immunoprecipitation with V5-tagged FANCD2 stably expressed in PD20 cells. When FLAG-Tip60 was immunoprecipitated with anti-FLAG antibody, FANCD2 was co-precipitated (Fig. 4C). For the reciprocal co-immunoprecipitation a corrected PD20 clone, PD20C1, expressing a C-terminal V5-tagged FANCD2 construct and showing normal levels of radial formation following MMC treatment, was used. V5-tagged FANCD2 was immunoprecipitated from whole cell extracts with a monoclonal anti-V5 antibody. Immunoblot analysis showed that Tip60 was bound to FANCD2, as was BRCA2, a previously noted interaction (34) (Fig. 4D).

Because overexpression of recombinant proteins can sometimes lead to compartmentalization artifacts, immunoprecipitation of endogenous FANCD2 from HEK293 fibroblast extracts was performed. Tip60 was pulled down with FANCD2 (Fig. 4E). We conclude that by several different strategies, Tip60 co-immunoprecipitates with FANCD2.

FANCD2 and Tip60 Co-localize in Damage-induced Foci—Treatment of cells with ionizing radiation, ultraviolet radiation, hydroxyurea, or DNA cross-linkers induces nuclear foci, thought to be centers of ongoing DNA repair (5, 35). FANCD2 has been identified in such foci, along with other repair proteins (34, 36). We tested whether Tip60 co-localizes in such foci with FANCD2. PD20C1 cells expressing FANCD2-V5His were grown on coverslips, treated with MMC, and fixed and immunostained with α -V5 and α -Tip60 antibodies. Untreated cells showed a nuclear speckled pattern of FANCD2 and Tip60, with substantial overlap of the respective signals for FANCD2 and Tip60. Treatment with MMC led to bright nuclear foci (Fig. 5), again with substantial overlapping signals. To address the question of whether Tip60 is required for the induction of ICL-induced FANCD2 foci, Tip60 was depleted in PD20C1 cells, and the cells were examined with and without MMC damage. As expected, depletion of Tip60 eliminated Tip60 foci after treatment with MMC; however, FANCD2 foci formed normally. Thus FANCD2 and Tip60 co-localize in ICL-induced foci, but Tip60 is not required for localization of FANCD2 to such foci.

Tip60 Depletion Sensitizes Cells to DNA Cross-links—Given the interaction of Tip60 and FANCD2, we investigated whether Tip60 acts in ICL repair by testing the effect of siRNA-mediated depletion of Tip60 on cell survival. Immunoblotting of whole cell extracts with anti-Tip60 antibody confirmed the depletion (Fig. 6A). Fibroblasts depleted for Tip60 showed pronounced sensitivity to MMC (Fig. 6B) in cell survival tests relative to control nonsense siRNA-transfected cells. After MMC treatment, formation of FANCD2-Ub was normal in cells

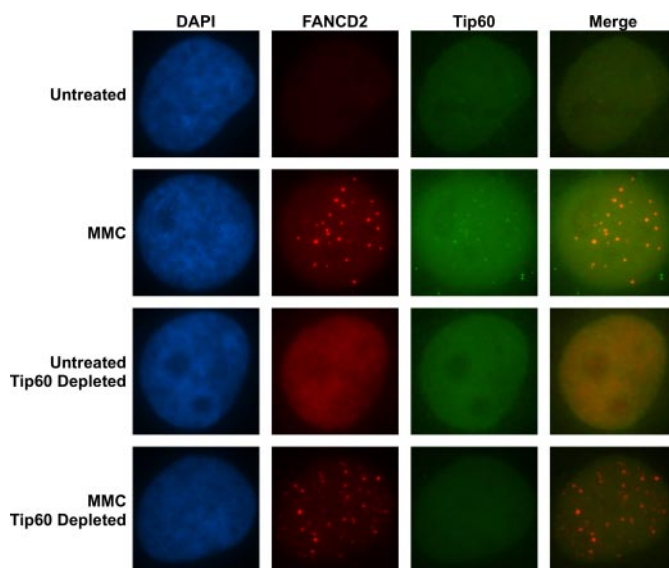


FIGURE 5. Co-localization of Tip60 with FANCD2 in nuclear foci after DNA damage. Upper panels, the corrected PD20C1 cells were either untreated or treated with 80 ng/ml MMC for 12 h, and the foci were visualized with anti-V5 (red) and anti-Tip60 (green) antibodies. Lower panels, Tip60 siRNA was used to deplete Tip60 in PD20C1 cells; cells were then either untreated or damaged with 80 ng/ml MMC for 12 h and visualized. DAPI, 4',6'-diamino-2-phenylindole.

depleted for Tip60 (Fig. 6C). Depletion of another component of the Tip60 chromatin remodeling complex, Tip49 (20), resulted in an increase in radial formation following MMC treatment (Fig. 6E and Table 2) as well as increased sensitivity to MMC in a survival test (Fig. 6F). Thus components of the Tip60 chromatin remodeling complex are required for normal ICL repair, but Tip60 is not needed for normal FANCD2-Ub formation.

A decrease in survival after ICL formation following Tip60 depletion might be due to alterations in the cell cycle secondary to the depletion. Cell cycle analyses with Tip60 depletion, with and without MMC treatment were unremarkable (data not shown). Tip60-depleted cells did not show a pronounced G₂ arrest after MMC treatment; an accumulation of cells in S phase very similar to mock-depleted cells indicated an intact S phase checkpoint. Thus depletion of Tip60 does not appear to alter the cell cycle significantly.

Tip60 Functions in the FA Pathway for ICL Repair—In yeast, there are three genetically distinct pathways for the repair of ICLs (37), and distinct ICL repair pathways exist in mammalian cells as well (29). We tested the epistatic relationship of Tip60 with FA by depletion of Tip60 in GM6914 (FA-A) and PD331 (FA-C) fibroblasts. Compared with nondepleted cells, Tip60-depleted PD331 cells do not show decreased survival after treatment with MMC (Fig. 6D). The same is true for GM6914 (not shown). In comparison with the results with normal fibroblasts, this indicates that Tip60 acts in the ICL response in the FA pathway. There is no increase in radial formation after MMC treatment in either FA-A or FA-C cells with Tip60 depletion (Table 2). Therefore by the criteria of cell survival and radial formation, Tip60 is epistatic with the FA pathway for ICL response.

Direct Interaction between FANCD2 and Tip60

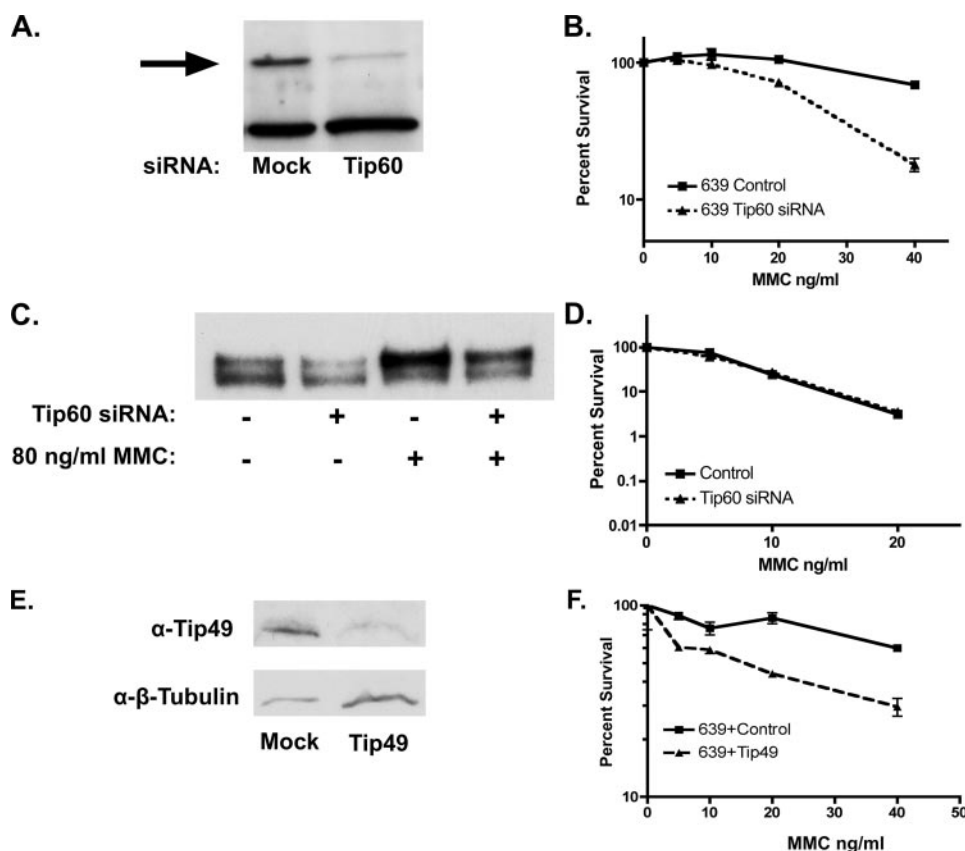


FIGURE 6. Depletion of Tip60 and Tip49. A, immunoblot showing Tip60 depletion 72 h post-transfection. The lower band is a nonspecific band that serves as a loading control. B, colony-forming assay with depletion of Tip60 and MMC treatment. C, Western blot of whole cell extracts, showing FANCD2-Ub formation following MMC treatment in Tip60-depleted GM639 cells. D, colony-forming assay with depletion of Tip60 in (FA-C) cells (PD331) and MMC treatment. E, immunoblot of mock-depleted and Tip49-depleted GM639 fibroblasts, 48 h post-transfection. F, colony-forming assay of GM639 cells with Tip49 depletion and MMC treatment.

TABLE 2
Radial formation with depletion of Tip60 or Tip49

	Radials
	%
GM639	1.0 ± 2.0
GM639, Tip60 siRNA	1.5 ± 1.0
GM 639 + 40 ng/ml MMC	23.0 ± 13.0
GM 639, Tip60 siRNA + 40 ng/ml MMC	23.5 ± 6.6
GM6914	6.7 ± 3.1
GM6914, Tip60 siRNA	4.0 ± 2.0
GM6914 + 5 ng/ml MMC	48.7 ± 7.0
GM6914, Tip60 siRNA + 5 ng/ml MMC	24.3 ± 8.6
PD331	2
PD331, Tip60 siRNA	17 ± 9
PD331 + 20 ng/ml MMC	92 ± 6
PD331, Tip60 siRNA, 20 ng/ml MMC	58 ± 18
GM639	0
GM639, Tip49 siRNA	4 ± 2
GM639 + 40 ng/ml MMC	26 ± 4
GM639, Tip49 siRNA, 40 ng/ml MMC	66.5 ± 8.5

DISCUSSION

A yeast two-hybrid screen using FANCD2 identified Tip60 as a FANCD2-interacting protein. The interaction in yeast is independent of the monoubiquitination of FANCD2, because a K561R mutation does not disrupt the interaction. However, mutations in the Tip60 acetyl-CoA-binding site do disrupt the Tip60-FANCD2 interaction, suggesting that the structure or occupancy of this site is important for the FANCD2 interaction.

This opens the possibility that FANCD2 or another FANCD2-interacting protein may be a novel target for Tip60 acetylation. The ability of Tip60 to acetylate ATM (24) and other nonhistones leaves open the question of where such acetylation might take place.

The findings presented here demonstrate that Tip60 does play a role in cell survival after ICL formation. Because FANCD2-Ub is localized to chromatin, it is reasonable to presume that the functional interaction of Tip60 and FANCD2 is in chromatin and that this leads to the action in a common pathway for ICL response. However, the intact monoubiquitination of FANCD2 in Tip60-depleted cells would imply that Tip60 acts “downstream” of FANCD2 ubiquitination, by the FA core complex.

It is not clear whether the Tip60-FANCD2 interaction *in vivo* involves either the Tip60 complex or the newly described “ID” complex involving FANCI and FANCD2 (8). The yeast two-hybrid interaction suggests that the interaction might occur in the absence of other associated proteins; however, the co-localization of Tip60 and FANCD2 in

damage-induced foci suggests that the interaction could occur while both proteins are participating in complexes in DNA repair centers. We depleted another member of the mammalian Tip60 complex, Tip49, and found that produced increased MMC sensitivity. Thus at least one other member of the Tip60 complex is required for ICL repair. These results are compatible with functional interaction requiring the entire complex but do not prove that.

Tip60 co-localizes with ATM in nuclear foci within 20 min after DNA damage, and the association of Tip60 with ATM is required for activation of ATM (24). Thus Tip60 may function as a protein acetylase, with a nonhistone protein being the target. Function of Tip60 downstream from FANCD2 monoubiquitination does not rule out such an acetylation of additional proteins of ICL repair. The recent report that Tip60 acetylates H2AX and that this is required for subsequent ubiquitination of H2AX by UBC13 may provide a clue (25). Tip60 is not required for FANCD2-Ub formation, needed for the FA pathway to act and for localization of FANCD2 to chromatin. Thus it would appear that modification of H2AX by Tip60 is not the required step for Tip60 in the FA pathway.

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